Remarks

Support for the Amendments and Status of the Claims

Support for the foregoing amendments to claims 1, 181-186 and 193 can be found throughout the specification, for example, at pages 28-29 and 33-38. Therefore, these amendments do not introduce new matter, and their entry and consideration are respectfully requested.

By the foregoing amendments, claims 1, 181-186 and 193 are sought to be amended. Claims 192 and 198 have been canceled without prejudice or disclaimer. Applicants retain the right to file one or more divisional or continuation applications directed to the subject matter of these canceled claims. Upon entry of the foregoing amendment, claims 1-16, 93-101, 106-107, 112-133, 138-159, 162, 164-165, 167, 169, 171, 173-175, 177, 181-186, 188 and 193-197 are pending in the application, with claim 1 being the sole independent claim.

Applicants note that, in the Office Action at the bottom of page 2, the Examiner has indicated that claims 192-197 are withdrawn from consideration. However, at the top of page 3, the Examiner indicates that claims 192-195 are currently under consideration, and in fact, has rejected claims 192-195 throughout the Office Action. Applicants respectfully submit that claims 12-16, 93-101, 106-107, 112-133, 138-140, 142-159, 162, 165, 167, 171, 175 and 196-197 have been withdrawn from consideration by the Examiner, and request that the Examiner confirm the withdrawn claims with the next Office Communication.

Summary of the Office Action

In the Office Action dated June 29, 2007, the Examiner has made nine rejections of the claims. Applicants respectfully offer the following remarks to traverse each of these elements of the Office Action. Applicants respectfully request reconsideration of the present Application.

Rejection Under 35 U.S.C. § 101

In the Office Action at pages 3-4, the Examiner has rejected claims 1-11, 141, 164, 169, 170, 173, 174, 177, 178, 181-186, 188, 192-195 and 198 under 35 U.S.C. § 101, as allegedly being directed to non-statutory subject matter. Applicants note that claims 170 and 178 were canceled in the Reply to Office Action filed on December 19, 2006. In addition, claims 192 and 198 have been canceled in the present Reply. Hence, this rejection is moot with regard to these claims. Applicants respectfully traverse this rejection as it may apply to the remaining claims.

The Examiner asserts that the claimed array would read on naturally occurring arrays of kinases, including kinases present in mammals bound to a membrane. The Examiner further asserts that there is no utility for the claimed arrays. Applicants respectfully disagree with the Examiner's assertions.

Applicants note that present claim 1 recites that the positionally addressable arrays comprise 61 purified active kinases or functional kinase domains thereof. Purified kinases or functional kinase domains thereof clearly cannot read on kinases in an *in vivo* setting or in any alleged form of naturally occurring array, as asserted by the Examiner. Purified kinases, for example, as discussed at page 29 of the specification, can be

purified using glutathione beads and standard protocols (though other methods can also be used). Purified kinases, as recited in the presently claimed invention, therefore clearly do not read on kinases in the body.

In addition, Applicants note that the present specification clearly provides numerous utilities for the presently claimed arrays at pages 17-26, include detection of various protein-protein interactions involved in disease states (e.g., patient screening), identification of cell surface markers, screening for various antibodies, etc. Hence, Applicants submit that the presently claimed invention clearly meets the utility requirements of 35 U.S.C. § 101.

In view of the foregoing remarks, Applicants respectfully request reconsideration and withdrawal of this rejection.

Rejection Under 35 U.S.C. § 112, First Paragraph, Written Description

In the Office Action at page 4, the Examiner has rejected claims 1-11, 141, 164, 166, 169, 170, 173, 174, 177, 178, 181-186, 188, 192-195 and 198 under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement. Applicants note that claims 170 and 178 were canceled in the Reply to Office Action filed on December 19, 2006. In addition, claims 192 and 198 have been canceled in the present Reply. Hence, this rejection is moot with regard to these claims. Applicants respectfully traverse this rejection as it may apply to the remaining claims.

The Examiner asserts that the term "comprises 61 kinases" is not supported in the as-filed specification, and that present claim 198 is also allegedly not supported. Applicants respectfully disagree with the Examiner.

The Examiner's attention is directed to the present specification, Example I at pages 27-38, which discloses the production of positionally addressable arrays comprising at least 122 yeast kinases. Applicants respectfully submit that the production of an array comprising 122 kinases clearly, must implicitly and inherently, encompass an array comprising 61 kinases. Furthermore, Applicants note the specification at page 11, lines 14-19, discloses the use of 50%, 75%, 90% or 95% of all of the expressed proteins with same type of biological activity in the genome of an organism. A person of ordinary skill in the art would readily recognize that 50% of 122 kinases is equal to 61 kinases, as recited in present claim 1.

Applicants note that there is no *in haec verba* requirement of the written description requirement of 35 U.S.C. § 112, first paragraph, and that newly added claim limitations may be supported in the specification through express, implicit, or inherent disclosure. *See* M.P.E.P. § 2163(I)(B). Applicants note that the preparation of an array comprising 122 kinases must, at the very least implicitly and inherently, provide support for an array comprising 61 kinases. Furthermore, disclosure of arrays comprising 50% of 122 kinases (or 61 kinases) provides explicit support for the recitation of 61 kinases in present claim 1. Thus, a person of ordinary skill in the art would readily understand that Applicants were in full possession of the presently claimed invention at the time of filing.

With regard to the Examiner's assertion that there is no support for present claim 198, Applicants note that this claim has been canceled in the present Reply. However, Applicants respectfully submit that the present specification clearly indicates that the kinases placed on the array clearly retain kinase activity (i.e. are active). As set forth in

Example I, at pages 33-34, an *in vitro* assay of kinase activity utilizing the prepared arrays demonstrated that the kinases exhibited kinase activity for at least one substrate. This clearly shows that the kinases retained their activity after be placed on the solid support (i.e., are active). Thus, a person of ordinary skill in the art would readily understand that Applicants were in full possession of the presently claimed invention at the time of filing.

In view of the foregoing remarks, Applicants respectfully request reconsideration and withdrawal of the written description rejection under 35 U.S.C. § 112, first paragraph.

Rejection Under 35 U.S.C. § 112, First Paragraph, Enablement

In the Office Action at pages 5-7, the Examiner has rejected claims 1-11, 141, 164, 166, 169, 170, 173, 177, 178, 181-186, 188, 192-195 and 198 under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the enablement requirement. Applicants note that claims 170 and 178 were canceled in the Reply to Office Action filed on December 19, 2006. In addition, claims 192 and 198 have been canceled in the present Reply. Hence, this rejection is moot with regard to these claims. Applicants respectfully traverse this rejection as it may apply to the remaining claims.

The Examiner contends that while the present specification is enabling for kinases from yeast, it does not provide sufficient enablement of an array comprising 61 kinases and functional kinase domains of mammal or Drosophila. The Examiner asserts that the presently claimed arrays encompass an enormous scope because the present claims do not place any limitations on the kind, number and/or length of kinase.

Furthermore, the Examiner indicates that the specification does not provide any reasonable assurance that the 61 kinases found in yeast could be found in mammals or Drosophila. The Examiner therefore concludes that the presently claimed invention is not enabled. Applicants respectfully disagree with the Examiner's contentions and conclusions.

As set forth in M.P.E.P. § 2164.01(a), there are many factors to be considered when determining whether there is sufficient evidence to support a determination that a disclosure does not satisfy the enablement requirement and whether any necessary experimentation is "undue." These factors include the breadth of the claims; the nature of the invention; the state of the prior art; the level of one of ordinary skill; the level of predictability in the art; the amount of direction provided by the inventor; the existence of working examples; and the quantity of experimentation needed to make or use the invention based on the content of the disclosure. *In re Wands*, 858 F.2d 731, 737, (Fed. Cir. 1988).

As noted by the Examiner, the present specification clearly provides an enabling disclosure of arrays comprising yeast kinases. Applicants note that the use of kinases from other organisms, including mammals and Drosophila, in the arrays of the presently claimed invention would not have required undue experimentation, but rather, simple, straightforward experiments. The protein kinases and functional kinase domains for use in the presently claimed invention are all well-known, well-characterized proteins that the ordinarily skilled artisan would easily comprehend. For example, as set forth in *Hanks, S.K. and Hunter, T., FASEB J.*, 9:576-596 (1995), the authors state that, as of 1995, "there are now hundreds of different members [of the kinase superfamily] whose

sequences are known." *Hanks and Hunter*, page 576. Furthermore, kinases, for example serine kinases, were already readily recognized in 1995 by virtue of their conserved subdomains. *Hanks and Hunter*, page 576 (abstract). In fact, in the Office Action dated July 31, 2006, Examiner Tran acknowledged that "the large protein kinase superfamily are well characterized and known in the art such that the sequence of *any* kinases from *any* mammal, yeast and Drosophila can be determine[d] by bioinformatics tools and publicly available sequence information." Office Action at page 7, lines 4-7 (emphasis added). Furthermore, methods that could be used to confirm kinase activity were well known as of the filing date of the present application (*see e.g.*, Example I of present specification). Thus protein kinases, and functional kinase domains thereof, were well-known in the art at the time of filing the present application.

Applicants submit therefore, that the state of the art in protein kinases at the time of filing of the present application was such that the ordinarily skilled artisan, possessing a typical level of skill in protein purification and analysis, would have readily recognized that the kinases of mammals, yeast and Drosophila could be readily identified. The presently claimed invention recites a positionally addressable array which comprises 61 purified active kinases or functional kinase domains of a mammal, yeast or Drosophila. Contrary to the Examiner's assertion at page 6 of the Office Action, the presently claimed invention does not require the arrays to comprise the *same* 61 kinases, but rather, to simply comprise at least 61 purified active kinases (or functional domains) of these organisms. As noted above, it was well within the ability of the ordinarily skilled artisan at the time of filing of the present application to identify which proteins of a mammal, yeast or Drosophila were protein kinases or functional kinase domains thereof, and

prepare an array comprising at least 61 of these purified active kinases or functional domains.

As noted above, other factors that are to be considered when determining whether the claims are enabled by the specification are the amount of direction provided by the inventor; the existence of working examples; and the quantity of experimentation needed to make or use the invention based on the content of the disclosure. Applicants note that the present specification clearly provides numerous examples of methods for preparing the presently claimed positionally addressable arrays utilizing yeast protein kinases (*see* specification at pages 27-38). Based on the knowledge available in the art at the time of filing, specifically, the ability to identify and prepare purified protein kinases from yeast, mammals and Drosophila, in combination with the detailed directions provided in the specification, it would not have required undue experimentation to prepare arrays comprising the kinases from any of these organisms.

The Examiner contends that, as the field of biotechnology is highly unpredictable, one cannot determine whether the generation of arrays comprising kinases of one organism (yeast) would be predictive of arrays comprising kinases of mammals or Drosophila. Applicants respectfully disagree with the Examiner. The present specification provides detailed methods for attaching kinases or functional kinase domains to the surface of a solid support (e.g., polydimethylsiloxane), for example, through the use of a 3-glycidooxypropyltrimethyoxysilane linker (GPTS). Applicants submit that the source of the kinase would not have any effect on the ability to attach the proteins to the surface of a solid support. The fact that a yeast kinase can be attached in

this manner would clearly provide sufficient guidance to a person of ordinary skill in the art to utilize the same methods for attaching a kinase from a mammal or Drosophila.

Applicants submit that, attaching mammalian or Drosophilian kinases to a solid support, using the detailed methods disclosed in the present specification, would clearly be within the abilities of the ordinarily skilled artisan. The Examiner is reminded that the test of enablement is not whether any experimentation is necessary, but whether, if experimentation is necessary, it is *undue*. *See* M.P.E.P. § 2164.01 (emphasis added). Applicants submit that at most, only minor, routine experimentation would be required to prepare arrays of mammalian or Drosophilian kinases or functional kinase domains. As noted above, kinases from yeast, mammals or Drosophila were easily identified and generated at the time of filing of the present application. Utilizing the detailed guidance provided in the present specification for attaching yeast kinases to a solid support, a person of ordinary skill in the art, with only routine experimentation, would have been able to prepare positionally addressable arrays comprising at least 61 purified active kinases or functional kinase domains thereof, any of the recited yeast, mammal or Drosophila organisms.

Accordingly, Applicants submit the present specification clearly enables the presently claimed invention. Reconsideration and withdrawal of this rejection are therefore respectfully requested.

Rejection Under 35 U.S.C. § 112, Second Paragraph

In the Office Action at pages 7-8, the Examiner has rejected claims 1-11, 141, 164, 166, 169, 170, 173, 174, 177, 178, 181-186, 188, 192-195 and 198 under

35 U.S.C. § 112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. Applicants note that claims 170 and 178 were canceled in the Reply to Office Action filed on December 19, 2006. In addition, claims 192 and 198 have been canceled in the present Reply. Hence, this rejection is moot with regard to these claims. Applicants respectfully traverse this rejection as it may apply to the remaining claims.

The Examiner states that "claim 1 is indefinite as to what determines a 61 kinase in one organism, let alone, in a plurality of organisms have 61 kinase especially the functional kinase domain of said kinase." Applicants note that the Examiner appears to consider "61 kinase" to be a single, unique kinase, while present claim 1 recites "comprises 61 purified *kinases*." Applicants respectfully submit that the ordinarily skilled artisan would readily understand that the phrase "61 purified active kinases, or functional kinase domains thereof," indicates that the array comprises at least 61 purified active kinases (or functional domains), but does not require any particular kinase to be present. There is no requirement that each organism possess the same single "61 kinase," or even the same 61 kinases, but rather, simply that the array comprise 61 purified active kinases (or functional domains) of the recited organisms. As noted above, identification of kinases from yeast, mammals and Drosophila was readily performed at the time of filing of the present application. Applicants respectfully submit therefore that the phrase 61 purified active kinases is neither vague nor indefinite.

The Examiner further contends that it is unclear whether the 61 kinase is present in one array or each spot in an array comprises 61 kinase from each different organisms. Applicants respectfully submit that the ordinarily skilled artisan would readily

understand, as recited in present claim 1, that the presently claimed arrays comprise a plurality of different substances on a solid support, *with each different substance being at a different position on the solid support*, wherein the density of the different substances on the solid support is at least 100 different substances per cm^2, and wherein the *plurality of different substances comprises 61 purified active kinases or functional kinase domains thereof*. Thus, as recited in present claim 1, each different position (i.e., "spot") on the array comprises a different substance, and the array comprises at least 61 kinases or functional kinase domains thereof. That is, each spot on the array comprises a different kinase or functional kinase domain. Applicants respectfully submit that the ordinarily skilled artisan would readily understand the scope of present claim 1, and hence, this claim is not indefinite.

The Examiner also asserts that the metes and bounds of the term "functional kinase domains" is not clearly set forth in the specification. Applicants respectfully disagree. The Examiner is directed to the specification at page 7, lines 14-19, where the term "functional domain" is defined. Applicants submit that, in view of this definition, as well as the use of the phrase throughout the specification, the ordinarily skilled artisan would readily understand the metes and bounds of the term "functional kinase domains," and hence, this phrase is not indefinite.

The Examiner further contends that claim 1 is indefinite for improper Markush claiming. Applicants respectfully disagree with the Examiner. However, Applicants note that present claim 1 recites, 61 purified active kinases or functional kinase domains thereof of a mammal, yeast, or Drosophila. Hence, this claim no longer utilizes a Markush grouping, and hence, this rejection has been rendered moot.

Finally, the Examiner asserts that it is unclear what is meant by the phrase "domains retain kinase activity." Applicants respectfully submit that the ordinarily skilled artisan would readily understand that this phrase is used to indicate that the kinases or functional kinase domains retain activity (i.e. are active) even though they have been purified and placed on a solid support, as set forth in the specification at pages 33-35. Hence, Applicants submit that this phrase is not indefinite.

In view of the foregoing remarks, Applicants respectfully request reconsideration and withdrawal of these rejections under 35 U.S.C. § 112, second paragraph.

Rejection Under 35 U.S.C. § 102(a), or 35 U.S.C. § 103(a), Over Uetz

In the Office Action at pages 8-10, the Examiner has rejected claims 1-11, 141, 181-186 and 192-195, as allegedly being anticipated by, or in the alternative, as allegedly being obvious in view of, Uetz et al., Nature 403:623-631 (February 10, 2000) (hereinafter Uetz). By the foregoing amendments, claim 192 has been canceled. Hence, this rejection has been rendered moot as it may have applied to this claim. Applicants respectfully traverse this rejection as it may apply to the remaining claims.

The Examiner contends that Uetz discloses a protein array comprising yeast genome encoded proteins, and that the proteins were expressed in 96-well plates. The Examiner asserts that the claimed kinase would have been inherent to the yeast array disclosed in Uetz, since yeast inherently contain kinase in their structure, or that they would have been obvious to determine given the identified genome of yeast. Applicants respectfully disagree with the Examiner.

As discussed above, present claim 1 (and hence, claims 2-11, 141, 181-186 and 193-195 that depend ultimately therefrom) recites a positionally addressable array comprising 61 purified active kinases or functional kinase domains thereof at a recited density. Applicants respectfully submit that Uetz does not disclose the preparation of an array comprising purified active kinases, and hence, cannot anticipate the presently claimed invention.

As set forth in the Methods section of Uetz, at page 627, the disclosed arrays were prepared by transferring patches of transformed yeast cells into wells of a micro-array assay plate. Uetz does not disclose any purification of the yeast proteins prior to placement in the assay plate, just simply transfer of the transformed cells. Hence, Uetz does not disclose the use of *purified* kinases or functional kinase domains, as recited in present claim 1. As set forth in M.P.E.P. § 2131, "A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." *Verdegaal Bros. v. Union Oil Co. of Cal.*, 814 F.2d 628, 631 (Fed. Cir. 1987). Thus, as Uetz does not disclose each and every element of present claim 1, it cannot and does not anticipate the presently claimed invention.

With regard to the Examiner's assertion that Uetz renders obvious the presently claimed invention, Applicants note that, even assuming the arrays disclosed in Uetz comprise 61 kinases, there is no disclosure in Uetz sufficient to render obvious the construction of an array of at least 61 kinases or functional kinase domains, in which the array comprises kinases that are *purified and active*, as recited in present claim 1.

As reaffirmed by the U.S. Supreme Court, courts are "to look at any secondary considerations that would prove instructive," when considering the obviousness of an

invention. *KSR Int'l. Co. v. Teleflex Inc.*, 127 S.Ct. 1727, 1739 (April 30, 2007). For example, as set forth in M.P.E.P. § 2141(III), objective evidence or secondary considerations such as unexpected results and the skepticism of experts is relevant to the issue of obviousness and must be considered in every case in which they are present. Furthermore, as noted in M.P.E.P. § 2143.02, evidence showing there was no reasonable expectation of success may support a conclusion of nonobviousness. *In re Rinehart*, 531 F.2d 1048, (CCPA 1976).

Applicants respectfully submit that, at the time of filing of the present application, it was unexpected that purified kinases and functional kinase domains of these kinases, could be purified and placed on a solid support to form an array, and that these kinases and kinase domains, *would retain their kinase activity*. It is only after the guidance provided in the present specification that a person of ordinary skill in the art would consider it possible to generate the presently claimed arrays.

Applicants submit herewith two references describing skepticism from those in the field regarding the preparation of protein arrays, even four years after the filing date of the present application. For example, at page 1129, left hand column, second full paragraph of Anderson, K.S. and LaBaer, J., *Journal of Proteome Research 4*:1123-1133 (March 30, 2005) (copy attached herewith as Exhibit A), the authors note:

> Their theoretical advantages notwithstanding, protein microarrays have still not found widespread use, in part because producing them is challenging. Historically, it has required the high-throughput production and purification of protein, which then must be spotted on the arrays. Once printed, concerns remain about the shelf life of proteins on the arrays.

Furthermore, at page 1, third paragraph line 3, through the fourth paragraph of Shaw, G., *Drug Discovery and Development* (February 3, 2005) (copy attached herewith as Exhibit B), the author notes:

> "It was first thought that protein biochips would just be an extension of DNA microarrays, and that hasn't exactly panned out," says Bodovitz.
>
> That's because proteins have proven to be much trickier to work with in array format than their genomic counterparts. First of all, there are issues of stability. Membrane proteins, for example, make up the majority of potential drug targets, but their particularly challenging to stabilize. Then there's the choice of immobilization technique, which determines how well the target protein presents itself to the capture agent, and the problem of nonspecific binding. And of course, proteins are inherently unstable outside their natural habitat of living cells, making them much more challenging than DNA to tag and manipulate.

Thus, as noted in the references cited above, Applicants respectfully submit that experts in the field were clearly skeptical of the ability to prepare protein arrays comprising purified active enzymes. Thus, there was no reasonable expectation of success of utilizing the methods disclosed in Uetz to prepare arrays comprising purified, active kinases on a solid support, at the time of filing of the present application. Only by following the teaching and guidance provided in the present specification could such arrays be produced. Thus, Applicants respectfully submit that the presently claimed invention cannot be rendered obvious in view of Uetz.

In view of the foregoing remarks, Applicants respectfully request reconsideration and withdrawal of the rejections under 35 U.S.C. § 102(a) and 35 U.S.C. § 103(a), over Uetz.

Rejection Under 35 U.S.C. § 103(a) Over Shalon, In View of Felder or Lafferty

In the Office Action at pages 10-12, Examiner has rejected claims 1-11, 141, 181-186, 188 and 192-195 under 35 U.S.C. § 103(a), as allegedly being upatentable over Shalon (WO 95/35505; hereinafter "Shalon") in view Felder *et al.* (U.S. Patent No. 6,458,533; hereinafter "Felder") of Lafferty (U.S. Patent No. 6,972,183; hereinafter "Lafferty"). By the foregoing amendments, claim 192 has been canceled. Hence, this rejection has been rendered moot as it may have applied to this claim. Applicants respectfully traverse this rejection as it may apply to the remaining claims.

The Examiner contends that Shalon discloses a microarray having regions with a density of at least about $100/cm^2$, and that the arrays can comprise enzymes. The Examiner notes, however, that Shalon does not disclose arrays comprising kinases. The Examiner relies on the disclosures of Felder or Lafferty to cure this deficiency. Specifically, the Examiner contends that Felder discloses that kinases are enzymes, and the Lafferty discloses an array containing substrate-enzymes, such as kinases. The Examiner therefore concludes that it would have been obvious to prepare the array disclosed in Shalon using the kinases disclosed in Felder and Lafferty, and hence, the presently claimed invention is rendered obvious. Applicants respectfully disagree with the Examiner's contentions and conclusions.

Applicants note that Shalon is primarily directed to arrays comprising polynucleotides (*see* Examples 1-3), and only mentions in passing that arrays comprising proteins and enzymes could be constructed. Furthermore, Felder discloses preparation of arrays comprising peptides that are *substrates* for kinases, not arrays comprising the kinases themselves, "[a] chimeric linker molecule is prepared in which a 25 base pair

oligonucleotide complementary to one of the anchors is crosslinked to a *peptide substrate of a tyrosine phosphokinase enzyme*." Felder at column 44, lines 18-21 (emphasis added). Thus, Felder does not disclose the preparation of arrays comprising 61 purified active kinases or functional kinase domains thereof, as recited in present claim 1.

With regard to Lafferty, Applicants note that the arrays disclosed therein are limited to enzymes expressed in expression library cells, and that Lafferty does not disclose the purification of these enzymes prior to placement on a solid support, as recited in the presently claimed invention. As set forth in Lafferty, at column 18, lines 1-14:

> The library comprises a plurality of recombinant clones, which comprise host cells transformed with constructs comprising expression vectors into which have been incorporated nucleic acid sequences derived from the DNA samples. One or more substrates and at least a subset of the clones is then introduced, either individually or together as a mixture, into capillaries (all or a portion thereof) in a capillary array. Interaction (including reaction) of the substrate and a clone expressing an enzyme having the desired enzyme activity produces an optically detectable signal, which can be spatially detected to identify one or more capillaries containing at least one signal-producing clone. The signal-producing clones can then be recovered from the identified capillaries.

Applicants respectfully submit, therefore, that Lafferty does not disclose arrays comprising 61 purified active kinases, as set forth in the presently claimed invention.

In view of the foregoing remarks, Applicants respectfully submit that Shalon, Felder and Lafferty, alone or in combination, do not disclose the presently claimed positionally addressable arrays, specifically, arrays comprising 61 purified active kinases or functional kinase domains thereof, as set forth in present claim 1. Specifically, the

references cited by the Examiner do not disclose preparation of purified kinases which are then placed on a solid support, as recited in present claim 1. Thus, Applicants submit that the Examiner has not set forth a *prima facie* case of obviousness, as there are clearly differences between the cited references and the presently claimed invention that have not been addressed by the Examiner.

Furthermore, as set forth in detail above, Applicants respectfully submit that there is was no reasonable expectation of success of preparing an array comprising at least 61 purified active kinases or functional kinase domains of these kinases, based on the references cited by the Examiner. Applicants respectfully submit that, at the time of filing of the present application, experts in the field were skeptical, and it was unexpected, that purified kinases and functional kinase domains of these kinases, could be placed on a solid support to form an array, and that these proteins would retain their activity. It is only after the guidance of the present specification that a person of ordinary skill in the art would be able to generate the presently claimed arrays.

In view of the foregoing remarks, Applicants respectfully submit that the disclosures of Shalon, Felder and Lafferty, alone or in combination, cannot render obvious the presently claimed invention. Hence, reconsideration and withdrawal of the rejection under 35 U.S.C. § 103(a) are respectfully requested.

Double Patenting Rejection

In the Office Action at pages 12-13, the Examiner has provisionally rejected claims 1-11, as allegedly being upatentable under judicially created doctrine of

nonstatutory double patenting, in view of copending Application No. 10/477,329 (hereinafter "the '329 Application").

The Examiner asserts that the subject matter of the present application is fully disclosed in the '329 Application. Applicants respectfully request that this rejection be held in abeyance until allowable subject matter is determined in the '329 Application and the presently claimed invention. At that time, Applicants may consider filing a Terminal Disclaimer.

Conclusion

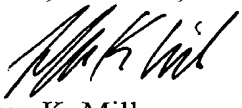
All of the stated grounds of rejection have been properly traversed, rendered moot or otherwise overcome. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding rejections and that they be withdrawn.

Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.



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The Sentinel Within: Exploiting the Immune System for Cancer Biomarkers†

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The release of proteins from tumors triggers an immune response in cancer patients. These tumor antigens arise from several mechanisms including tumor-specific alterations in protein expression, mutation, folding, degradation, or intracellular localization. Responses to most tumor antigens are rarely observed in healthy individuals, making the response itself a biomarker that betrays the presence of underlying cancer. Antibody immune responses show promise as clinical biomarkers because antibodies have long half-lives in serum, are easy to measure, and are stable in blood samples. However, our understanding of the specificity and the impact of the immune response in early stages of cancer is limited. The immune response to cancer, whether endogenous or driven by vaccines, involves highly specific T lymphocytes (which target tumor-derived peptides bound to self-MHC proteins) and B lymphocytes (which generate antibodies to tumor-derived proteins). T cell target antigens have been identified either by expression cloning from tumor cDNA libraries, or by prediction based on patterns of antigen expression ("reverse immunology"). B cell targets have been similarly identified using the antibodies in patient sera to screen cDNA libraries derived from tumor cell lines. This review focuses on the application of recent advances in proteomics for the identification of tumor antigens. These advances are opening the door for targeted vaccine development, and for using immune response signatures as biomarkers for cancer diagnosis and monitoring.

Keywords: tumor antigen • antibody • protein array • proteomics • tumor immunology • biomarkers

Introduction

The challenge faced by our immune system resembles that of an intelligent security system, which must continually monitor for the presence of foreign invaders, while recognizing and disregarding normal self. Like a vigilant sentry, immunologic memory persists long after exposure to the threat has abated. Recognizing the value of this persistent response, clinicians have exploited it for years to test individuals for current or past exposure to a wide variety of infections. Compared to other serum-derived proteins, antibodies are stable, highly specific, and readily detected with well-validated secondary reagents, making them ideal for such tests. Indeed, the traditional "blood test" required of couples before obtaining a marriage license is nothing more than a test for antibodies to the spirochete *T. pallidum* that causes syphilis. Thus, assessing immune responses is one of the oldest and most successful forms of biomarkers in medicine.

The immune system employs complex mechanisms to distinguish between self and nonself. It deletes or renders tolerant any cells which react to the constant stream of benign macromolecules in routine circulation. The system is not foolproof, however, and in certain diseases, the immune system responds to self-derived antigens, perhaps because their location, abundance, modified form, or other features appear unfamiliar. Cancer patients often produce responses to self-proteins that are expressed by their tumors, called tumor antigens, most of which are altered in some form that renders them immunogenic. These proteins may be unique to cancer and germ cells (the "cancer-testis" antigens), found only in specific tumors (prostate-specific antigen)[1] or in most tumors (telomerase).[2] They may be mutated (p53),[3] misfolded,[4] overexpressed (NY-ESO-1),[5] aberrantly degraded,[6] or aberrantly glycosylated (MUC-1).[7] The magnitude of the immune response to cancer, in general, is lower than the immune response to infectious agents and the potential number of tumor antigens encompasses the entire tumor proteome in all its variations. At present, we have a limited understanding of the breadth, extent, impact, and dynamic variation of the immune response to cancer (the "cancer immunome"). Identifying the specific targets of B- and T-lymphocyte immunity to cancer may (1) identify potential biomarkers for cancer diagnosis, classification, and monitoring of response, (2) determine the impact of immune regulation on cancer progression, and (3) identify potential antigens and mechanisms for immunotherapy development.

† Part of the Biomarkers special issue.
‡ Dana-Farber Cancer Institute.
§ Harvard Institute of Proteomics.

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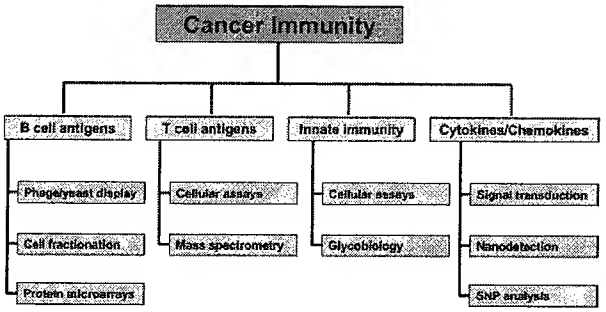

Figure 1. The application of cancer proteomics to tumor immunology. The immune response to cancer, whether endogenous or driven by immunotherapy, involves a complex array of interactions between the tumor, host microenvironment, lymphocytes, antigen presenting cells, antibodies, cytokines and chemokines. Major arms of the immune system are shown in blue, and selected techniques for evaluation are shown in green. The identification of target antigens of B- and T-lymphocyte recognition may be identified on a proteome-wide basis, through cell fractionation, protein microarrays, nanospray mass spectrometry, and protein expression profiling of tumors in vivo. Cellular assays may use protein microarrays, microfluidics, and nanotechnology to identify target antigens as well as host responses. Microenvironmental interactions can be analyzed, as well as signal transduction pathways and costimulatory/regulatory molecules. This review will focus on B-cell and T-cell target antigen identification.

The natural immune response is achieved through a tightly regulated, yet flexible network including antibodies, antigen presenting cells, T lymphocytes, cytokines, chemokines, regulatory systems, as well as microenvironmental signals (Figure 1). Of these responses, the targeted responses to protein (and carbohydrate) antigens relies on the development of antibodies and/or T lymphocytes to target epitopes. T lymphocytes can respond to antigens derived from within cells and without. They primarily recognize short peptides (8–22mer) derived from intracellular proteins (i.e., viral antigens) bound to self-MHC molecules for presentation to $CD8^+$ T lymphocytes. Exogenous antigens are endocytosed, degraded, and presented to $CD4^+$ lymphocytes (Figure 2). Antibody responses increase antigen presentation by enhancing uptake through the Fcγ receptors on antigen presenting cells. As a result, antibody targets may contain epitopes that are also recognized by T lymphocytes. This has formed the basis for using antibody responses to identify T cell antigens for immunotherapy.

Lessons from Autoimmune Diseases. Spontaneous autoantibodies were first identified in a series of clinical disorders in which the patients' immune systems mount a vigorous response to self-antigens, in some cases leading to debilitating symptoms. Systemic lupus, myasthenia gravis, rheumatoid arthritis, and others all involve this process, called autoimmunity. In these illnesses, the titers of autoantibodies often track with the severity of the illness and thus have long been used as serum biomarkers (reviewed in refs 8,9).

Although post hoc arguments can usually be constructed explaining why proven autoantigens may have appeared unfamiliar to the immune system, in general, we do not understand enough about the characteristics that determine whether a protein will act as an autoantigen to predict them a priori. To date, only one or two percent of proteins have been identified as autoantibody targets.[10] In autoimmune diseases, the autoantigens identified, such as Ro/SSA, La/SSB, Sm, RNP,

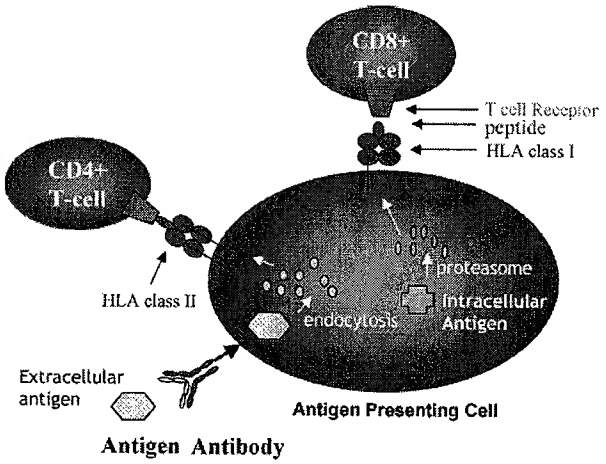

Figure 2. Classical model of antigen presentation. Intracellular antigens (i.e., viral) are cleaved into peptides by the proteasome and bound to MHC Class I molecules at the surface of cells for presentation to $CD8^+$ T lymphocytes. Exogenous antigens are endocytosed by antigen presenting cells (APC), which is markedly enhanced by binding of serum antibodies both to antigen and to the Fcγ receptor on the APC, with subsequent degradation and binding of peptides to MHC Class II molecules for stimulation of $CD4^+$ T lymphocytes. Because of this coordinated immune response, tumor antigens that cause antibody responses in patient sera may contain immunogenic T cell peptide epitopes as well (modified from *D. Miklos*).

Scl-70, and Jo-1 are primarily intracellular antigens that function in large complexes with nucleic acids involved in protein synthesis,[8] and are thought to arise as a result of overexpression, apoptosis, reduced degradation, or similarity to cross-reacting foreign antigens (mimicry). It is often not clear whether these autoantibodies have any direct role in disease pathophysiology. In contrast, antibodies directed at membrane proteins, such as the target antigens of hemolytic anemia or the acetocholine receptor in myasthenia gravis may contribute to the disease process through complement activation, antibody-dependent cytotoxicity, or interference with receptor/ligand interactions. Certain structural motifs enhance antigenicity, such as carbohydrates, multivalency, epitope repetition, and coiled-coils.[11] Some of these antigens are linked to degradation during apoptosis,[12] or are targets of granzyme B cleavage during T lymphocyte-induced cytolysis.[13,14] Further elucidation of the mechanisms underlying autoantibody generation will assist in predicting and identifying target antigens.

p53 as a Model Tumor Autoantigen. Perhaps the most well-studied autoantigen in cancer is the p53 protein (reviewed in [3]). In 1979, DeLeo et al.[15] demonstrated that autoantibodies to some tumor cells in mice were directed against the p53 protein. Subsequent studies have demonstrated that the half-life of mutated p53 (several hours) was markedly increased compared to wild-type (several minutes) resulting in accumulation in the cell nucleus. p53 autoantibodies are dependent on the type of p53 mutation.[16–18] Notably, the immunogenic epitopes have been mapped primarily to both the N- and C-terminal portions of the molecule, which are heavily glycosylated, but not to the central portion of the molecule, which harbors the mutations, suggesting that the accumulation of protein, rather than the mutations per se, results in autoantibody generation. Multiple assays using recombinant antigens and p53-derived peptides

have shown that some antigenic determinants are dependent on full-length antigen, conformation, or phosphorylation.[3,19]

The detection of p53 autoantibodies in serum correlates strongly with cancer ($p < 10^{-4}$). p53 autoantibodies have been studied in over 9489 patients with a wide variety of tumors.[3] Despite the strong specificity of the response, only 20−40% of patients with cancers harboring p53 missense mutations will have p53 antibodies in their sera.[3] Therefore, there are additional unknown factors beyond antigen accumulation that impact the development of autoantibodies. This illustrates one of the major challenges facing the use of single autoantibody tests for cancer detection. Although the appearance of such antibodies is highly suggestive of cancer, many patients will be missed because they do not respond to p53. p53 autoantibodies have been detected in body fluids, such as ascites, pleural effusions, and saliva, and these correlate with serum autoantibodies.[19−21] Interestingly, gliomas, which are associated with p53 mutations, are not associated with the development of p53 antibodies,[22,23] suggesting either that development of tumors in the CNS leads to immunologic privilege, or the use of steroids in this patient population could dampen the serologic immune response.

Antitumor Effects of Autoantibodies in Cancer. It is not known if autoantibodies reflect underlying immunosurveillance of cancer or have an impact on the clinical outcome of the disease. For example, multiple studies have attempted to correlate p53 autoantibodies with prognosis showing mixed results.[3] As the development of B cell immunity often depends on concordant T cell immunity, autoantibody identification can lead to the identification of relevant T cell antigens.[24−26] In patients who have undergone autologous tumor vaccination, certain autoantibodies correlate with tumor response to treatment.[25,27] In bone marrow transplantation, the development of autoantibodies may identify minor histocompatibility antigens associated with graft versus host disease[28] or tumor antigens associated with the graft-vs-leukemia (GVL) effects of donor lymphocyte infusion.[29,30] In murine tumor models, it is the coordinated activities of $CD4^+$ and $CD8^+$ T cells, CD1d-restricted invariant NKT cells, and antibodies that accomplish protective immunity.[31−34]

The serologic identification of tumor antigens has directly led to the development of a number of cancer vaccines currently in clinical trials. The proteinase-3 antigen, first identified as the target antigen of the ANCA antibody assay for Wegener's granulomatosis, is being tested as a target for vaccination in CML.[35] NY-ESO-1 has been used as an immunogen in multiple clinical trials.[36] Antibody response to tumor vaccines have been shown to correlate with improved disease-free and overall survival in stage II melanoma patients vaccinated with a polyvalent vaccine.[37] Melanoma patients vaccinated with autologous tumor cells may develop highly individualized antibody responses.[38] The identification of these autoantibodies has directly led to mapping of $CD4^+$ and CD8+ T lymphocyte epitopes. The clinical benefit of antigen-specific tumor vaccination strategies is just beginning to emerge, with the recent demonstration of a survival benefit of a dendritic cell-based vaccine in hormone-refractory prostate cancer.[39]

Autoantibodies as Biomarkers of Cancer. In 2003, it is estimated that 1 334 100 people in the US were newly diagnosed with cancer, with an estimated 556 500 patients dying of the disease.[40] In this setting, there is intense effort in the search for biomarkers that can predict disease, identify biologic subtypes of disease, track response to therapeutic interventions, predict side effect profiles, and monitor for disease progression and recurrence.[41]

There are a limited number of serum protein biomarkers that are widely used in clinical oncology for prognosis and treatment monitoring, and their use in early cancer diagnosis has been hampered by false-positive rates in the normal population. The identification and development of these biomarkers took decades of research and required large prospective trials. Carcinoembryonic antigen (CEA), widely used for the monitoring of adenocarcinomas, was originally identified from tumor lysates because of its immunogenicity in rabbits.[42] The subsequent identification of the prostate specific antigen (PSA)[43] and the ovarian cancer biomarker CA-125,[44] among others, have demonstrated both the validity and limitations of serum biomarkers in the diagnosis of cancer. Although PSA is now routinely used for screening healthy populations for prostate cancer diagnosis, most serum biomarkers are used for monitoring treatment or for screening highly selected high-risk populations. More recent developments in serum proteomics have been reviewed in this issue and elsewhere.[45]

Compared with other polypeptides, autoantibodies have many appealing features as biomarkers. First, although tumor antigens may circulate only briefly or in low concentration, perhaps due to transient shedding by tumors, rapid degradation in the serum, or rapid clearance, the corresponding antibody response is likely to be persistent. Second, antibodies are highly stable in serum samples and are not subject to the types of proteolysis that are commonly observed for other polypeptides (discussed elsewhere in this issue), making sample handling much easier. Third, the $t_{1/2}$ of antibodies in circulation is >7 days, so hourly or daily fluctuations are expected to be minimal, simplifying sample collection. Finally, the biochemical properties of antibodies are well understood and there are many available reagents for their detection, simplifying assay development. Given these advantages, the challenge that remains is demonstrating that antibodies can be sufficiently informative to reliably detect cancer.

Early Disease Diagnosis. Very limited data are available on the use of autoantibodies to reveal early disease. Antibodies have been detected as early as several years before the clinical appearance of cancer[46,47] and in patients with preneoplastic disease.[48] Anecdotal studies have detected p53 autoantibodies in heavy smokers prior to the diagnosis of lung cancer.[47] Another study showed p53 autoantibodies in individuals exposed to vinyl chloride, a risk factor for the development of angiosarcoma of the liver.[46] The p53 autoantibodies predated the diagnosis of angiosarcoma by several years. Although encouraging, these few studies are not yet sufficient to endorse the value of antibodies in the early detection setting.

Monitoring Treatment Response and Predicting Recurrence. Antibodies to tumor antigens have been detected in early stages of disease and fluctuate with tumor response. In patients with both stage III and stage IV neuroblastoma, 10% have evidence of antibodies to the NY-ESO-1 antigen, but the antibody is not seen in the sera of patients in clinical remission or in earlier stages of disease.[49] In melanoma, antibodies to TA90 antigen have been detected in only 12% of patients with 1−2 mm primary, node negative melanoma who subsequently relapsed, but 62% of case-controlled patients who did not.[50]

Antibodies to the her2/neu tumor antigen have been detected in the sera of 20% of patients with her2+ early-stage breast cancer[51] but only 7% of late-stage breast and ovarian cancer patients.[52] Some of the early-stage patients had titers

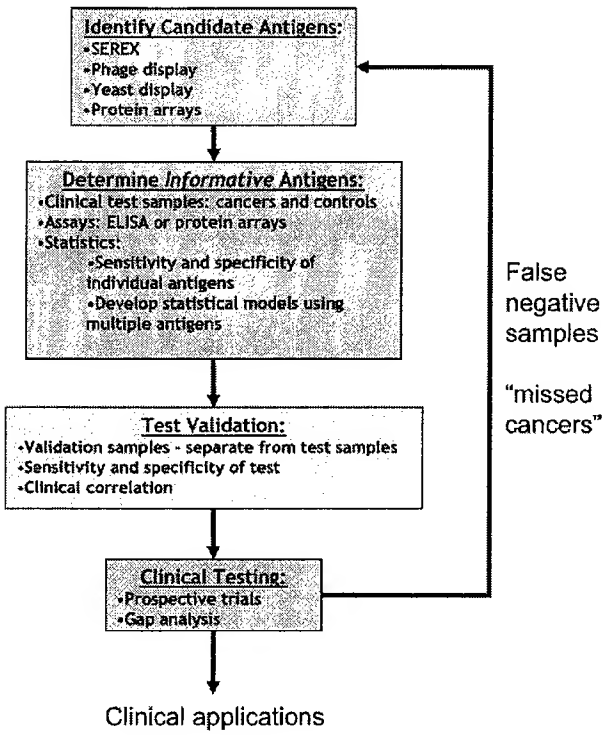

Figure 3. The development of autoantibody biomarkers in cancer. The identification of target antigens using antibodies derived from patient sera is the first step in identification of potential biomarkers for cancer diagnosis or immune monitoring. In the field of autoimmunity, detailed evaluation of the autoantibody quantity (titration) and specificity has required development of a confirmatory ELISA using heterologous recombinant protein. Antigens that segregate patient sera from control sera ("informative antigens") would be tested further. Analogous to DNA microarrays, well-annotated and blinded test sets and validation sets of sera (such as sets of sera from different stages or types of cancers) are required to identify areas of potential clinical applications. Finally, prospective clinical trial analysis is required to further validate the sensitivity, specificity, and predictive value of the assay. For tumor antigens, gap analysis that identifies false-negative sera ("missed cancers") would then be further screened for additional autoantibody biomarkers. The potential clinical applications of these biomarkers include diagnosis, immune monitoring, vaccine development, and endpoint analysis of cancer therapeutics.

>1:5000, suggesting a strong, possibly protective immune response.

The titer of antibodies to NY-ESO-1 have been shown to correlate with disease progression, as well as with disease response/resection.[53] The titer of anti-p53 antibodies have been shown to increase in a subset of patients with early-stage breast cancer prior to disease relapse,[54] and disappearance of anti-p53 antibodies has been observed in 27 patients after resection of colorectal cancer.[55] Tumor antigen/antibody immune complexes have been detected up to 19 months prior to the development of clinical relapse and correlate with survival in patients with early-stage melanoma.[56]

Methods for Assessing Autoantibodies. The development process for autoantibodies as biomarkers is outlined in Figure 3. Historically, this cycle begins with identifying antigens that are detected by the sera of cancer patients. Antigens must then be selected and tested by comparing sera from both patients and healthy donors to determine if the antigens are "informative", i.e., responses are limited to patients. As part of this process, the sensitivity and specificity of the antigens should be determined. Promising antigens are then tested on a set of samples separate from the training set to validate their usefulness. Ultimately, prospective trials will establish their value as biomarkers. The cycle is completed by using the sera from patients not detected by the test to screen for new antigens. In some of the newer proteomic approaches, two of these steps may combine into a single step.

Identification of Novel Tumor Antigens. SEREX. A variety of techniques have been developed that use patient sera as probes against candidate antigens derived from tumor cells and tumor cell lysates to find novel autoantigens. Prominent among these, serologic expression cloning (SEREX) was developed 10 years ago (Figure 4). This method uses patient sera to probe blotted phage expression libraries derived from tumor cells and has resulted in the identification of over 2000 autoantigens recognized by patient sera.[5,57-79] Through a database established by the SEREX collaborative group, sequences from over 1390 genes have been deposited[80] at http://www2.licr.org/CancerImmunomeDB/. As SEREX relies on immunoblotting, these antigens are limited to linear epitopes and those gene products that can be expressed in bacteria.

As with the autoimmune targets, most of the antigens identified with SEREX are nuclear or intracellular antigens, many of which are upregulated, mutated, or specifically expressed in tumors. However, altered degradative pathways, such as sensitivity to granzyme B cleavage, has been shown to have a marked impact on the immunogenicity of a hepatocellular tumor antigen.[6] In addition, frameshifts and alternative reading frames, can result in autoantibodies. Using colon cancer sera, the antigen ADO34 was identified with a frameshift insertion,[81] and CDX2 had a frameshift mutation.[82] Antibodies to the OGFr protein can recognize an alternative-reading frame of the molecule.[79]

The SEREX method identifies autoantigens whether or not they are informative. At present only a handful of antigens have undergone comparison testing, in part because of the technical challenges associated with historical assays for validating autoantigens.

Phage Display. Combinatorial phage display is a new and attractive approach for the identification of tumor antigens. By expressing antigens as fusions with phage proteins, antigens can be detected more rapidly and with less serum. Rather than immunoblotting as with SEREX, phage display relies on successive rounds of immunoprecipitation of phage libraries using patient serum. This powerful approach has been used in the field of rheumatology to identify autoantigens in multiple sclerosis, lupus, rheumatoid arthritis, and others.[83-85] For tumor antigens, one study showed that only 4 of 13 antigens identified with prostate cancer sera had already been identified by SEREX technologies, demonstrating that novel tumor antigens may be identified with different screening approaches.[86] Phage display has been successfully used to identify tumor autoantigens in cancer.[86-89] However, antigens expressed by phage display may not be in native conformation, do not have mammalian post-translational modifications, and each positive phage clone must be individually sequenced. Yeast display systems are also being investigated.[90]

Validating Autoantibodies. To be useful as biomarkers, the tumor antigens must distinguish between individuals with and

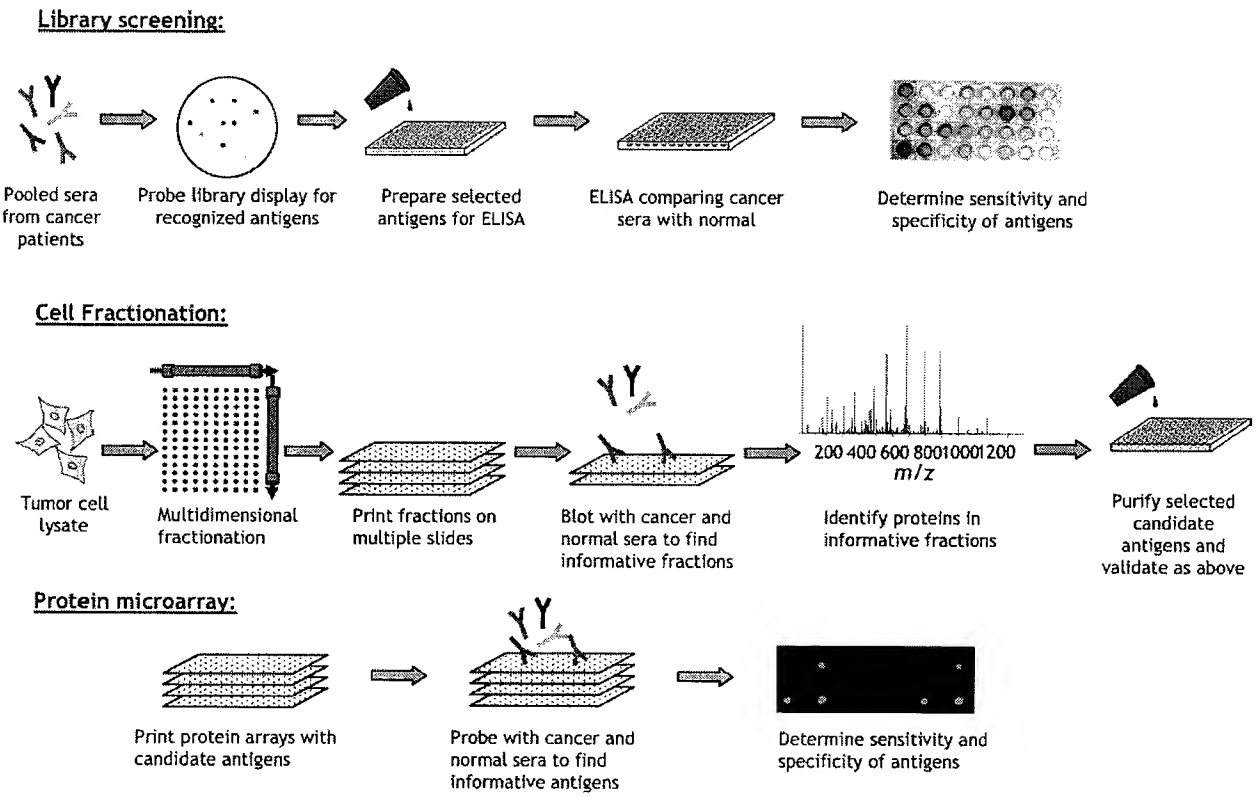

Figure 4. Methods of antigen identification. In traditional library screening (top row), cDNA libraries from tumor cells are expressed by phage, blotted on a membrane, and probed with patient sera. Confirmation of sensitivity and specificity requires recombinant expression of protein for ELISA analysis. Similar approaches of library screening may use phage display, yeast display or peptide libraries. Cell fractionation approaches (middle row) are a modification of the traditional western blot. Tumor cell lysates may be fractionated using microfluidic 2-dimensional or 3-dimensional separations, followed by printing of fractions on microarrays for probing with patient sera. Fractions identified in patient sera but not normal sera ("informative fractions") are then further evaluated by mass spectrometry to identify target antigens, which then must be confirmed for sensitivity and specificity using ELISA or similar methods. In the bottom row, protein microarrays can be printed using recombinant proteins or proteins translated in situ, then probed with patient sera. Because the protein microarrays are addressable, no further antigen identification is required, and the sensitivity and specificity of multiple antigens may be evaluated in parallel with screening.

without cancer. Thus, once a candidate autoantigen is identified, comparisons must be made regarding the responses of individuals from both groups. The measurement of an autoantibody response is most often accomplished using the enzyme linked immunosorbent assay (ELISA), in which purified protein (the autoantigen) is immobilized in the wells of a microtiter dish and exposed to serum samples (Figure 4). After adequate washing, any bound antibody is revealed using standard anti-human antibody reagents linked to enzymatic markers. If the response is very strong, then it may be useful to perform serial dilutions of the serum to determine the titer of the antibody. By comparing multiple patient and normal donor samples it is possible to determine the frequency of response. For example, antibodies to dsDNA are present in only 40−60% of SLE patients, but antibodies to histones are present in >95% of patients with drug-induced SLE. To compute the sensitivity and specificity of the test, a threshold value must be set, such that the test is considered positive if the antibody response achieves that value or above. Typically, this threshold is either 2 or 3 standard deviations above the average response in the normal population.

The development of clinical ELISAs require the use of recombinant proteins or peptides, which may be complicated by batch-to-batch variations, loss of conformational epitopes or lack of mammalian post-translational processing.[91,92] Antigenic epitopes from proteins such as Ro, La, SmB, SmD, Scl-70, can be linear epitopes of 10−22 amino acids conformational, or cryptic. Furthermore, epitopes may be naturally post-translationally modified by multiple means, including phosphorylation, glycosylation, acetylation, and methylation (SmD1, SmD3, fibrillarin, nucleolin),[93,94] all of which must be considered prior to the development of a clinical diagnostic assay. Of the many candidate antigens identified so far, only a handful have gone through the validation process to determine their sensitivity and specificity as cancer predictors, in part because of the challenges associated with setting up ELISAs.

Proteomic Approaches for the Identification of Tumor Antigens. The Power of Multiplexing. With all of their potential advantages, the Achilles heel of autoantibodies as biomarkers is their sensitivity, i.e., the fraction of true positives that have a positive test. In large part, this may reflect the nature of cancer. Unlike infections in which the vast majority of patients respond to the same immunodominant antigens, even cancers

Table 1. Current Methodologies for the Detection of Autoantigens

method	advantages	disadvantages	application
SEREX	Many clones, clone for gene automatically available	Denatured epitopes, laborious, large amounts of serum, requires separate validation step	Antigen discovery
Western Blotting	Reproducible	Large amounts of serum, requires separate steps to identify and validate antigens	Antigen Discovery
Phage display	Large number of clones, little serum required, ability to refine selection with multiple rounds, clone for gene automatically available	Partial gene products, gene overrepresentation, requires separate validation step	Antigen discovery
Cell Fractionation	Retains post-translational modification, multiple arrays allow for screening for fractions that are informative for cancer, do not need to have cloned gene or library for screening	Tedious, must identify the relevant antigen in potentially complex fraction, may need to clone gene for identified protein, requires separate validation step, post-translational modification may not be preserved in the validation step	Antigen discovery
ELISA	Reproducible, useful for comparing many serum samples	Tedious to produce protein for the assay.	Validation
Candidate antigen protein microarrays	Screen many candidate antigens, early validation and antigen discovery can be done simultaneously, very little serum required, clone for gene automatically available	Arrays can be challenging to produce, novel technology	Antigen discovery and validation

of the same type represent a mix of different biological subtypes. Thus, patients are likely to mount immune responses to different tumor antigens, and no single antigen is likely to detect all cancers. Typically, only 15−20% of patients demonstrate a response to any given antigen. However, proteomics may hold the key to success because it provides the means to multiplex. By linking the responses to several antigens together, the sensitivity and specificity of the test increases considerably, presumably because the chance that a patient will respond to at least one of the antigens is increased.[76,95−102] Koziol et al. demonstrated that a panel of just seven tumor-associated antigens (myc, cyclin B1, p62, IMP-1, Koc, p53, and survivin) could be used to segregate sera from patients with cancers from healthy donor sera.[95] In that study, sera from breast, colorectal, gastric, hepatocellular, lung, and prostate cancers were distinguished with sensitivities from 0.77 to 0.92 and specificities from 0.85 to 0.91 using no more than 3 tumor antigens for any cancer cohort. Scanlan et al. studied the serologic responses to 13 defined tumor antigens in sera from colon cancer patients. Of these, 46% of patients, but not healthy donors, had antibodies to at least 1 of these antigens.[103]

These results demonstrate the potential power of simultaneously analyzing multiple autoantigens. It provides a greater likelihood of detecting and diagnosing the appropriate cancer presumably because the sensitivity will improve (increased chance that the patient will have a response to at least one of the tested antigens) and the specificity improves (positive responses on multiple antigens increases certainty). By coupling this with the appropriate statistical modeling, it is likely that *patterns* or weighted schemes of antibody responses, rather than individual responses, will have the greatest utility in clinical assays. The early experiments above were performed by using recursive partitioning. Given the technical challenges inherent in that method for large numbers of antigens, newer technologies are needed for high throughput analysis. To this end, recent development of several proteomic technologies have been adapted for tumor antigen identification and biomarker development. These include probing fractionated tumor cell lysates, phage display, and protein microarrays (See Table 1).

Probing Fractionated Tumor Cell Lysate Blots with Serum. In this approach, tumor cell lysates are fractionated to separate the various protein species and blotted onto a membrane or microarray and then probed with patient or control sera. Response patterns are then analyzed to differentiate between the two (Figure 4). This has been used to demonstrate antibodies in the sera of lung cancer patients.[96,104] A similar approach was used to distinguish sera from prostate cancer patients from healthy donor sera with 98% accuracy.[105] The development of automated separation systems using liquid-based chromatography with subsequent microarray spotting of lysate fractions enhances the reproducibility and speed of immunogenic fraction identification. Advantages of this approach include the ability to query a large fraction of the tumor cell proteome and the preservation of the post-translational modifications of proteins. Moreover, when this is executed using a microarray format, only small amounts of serum are required. However, the reproducibility demanded by a clinical assay requires an identified and validated antigen. Thus, as with other chromatographic separation systems, the identity of the proteins in the

fraction must eventually be determined, presumably using sensitive mass spectrometric analysis. As each lysate fraction may contain many proteins at very different concentrations, of which the minor component may be the immunogen, antigen identification can be difficult.

Probing Candidate Antigen Arrays. Known or predicted tumor antigens may be directly spotted on microarrays and probed with human sera, with the advantage of reproducibility and more rapid screening of small amounts of sera (Figure 4). In this case, the identity of a single protein at each feature on the array is known a priori. This approach has been successfully used to screen autoimmune patient sera.[100,101] A powerful advantage of this approach is that it offers the ability to screen for informative autoantigens by comparing responses of patients to controls. Because each feature of the array represents a single protein, whose identity is known, it is possible to calculate sensitivity and specificity values for the response to each candidate antigen during the screening phase. This allows the rapid determination of which antigens are informative for cancer detection. Ultimately, focused arrays of tumor antigens previously identified by other means may have the greatest utility for immunodiagnostics.

Their theoretical advantages notwithstanding, protein microarrays have still not found widespread use, in part because producing them is challenging. Historically, it has required the high-throughput production and purification of protein, which then must be spotted on the arrays. Once printed, concerns remain about the shelf life of proteins on the arrays. Recently, programmable protein microarrays that consist of anchored cDNA's and in situ transcription and translation of tagged proteins have been shown to result in highly reproducible protein arrays without the requirement of heterologous protein expression and purification.[106] As the proteins are synthesized at the time of the assay, shelf life is not an issue and the proteins are translated with a mammalian reticulocyte lysate, which reproduces the folding and some of the post-translational processing of antigenic epitopes. Unlike tumor lysate fractionation, antigen identification is automatic, although epitopes that depend on abnormal processing by tumor cells would not be identified, and transmembrane proteins have not yet been tested in this system. Like most antigen presentation methods (SEREX, ELISA, phage display, etc.), these programmable arrays require access to cDNAs to express the antigens. Fortunately, nearly all known tumor antigen genes are available because they were identified with methods that involved cDNA identification. Moreover, libraries of cDNA clones representing most of the human proteome are increasingly available.

Proteomics and the Identification of T Cell Antigens. In contrast to the rapid identification of B cell antigens, the identification of T cell antigens remains much more difficult. Since T lymphocytes specifically recognize peptides derived from protein antigens, the isolation and confirmation of these antigens have traditionally relied on laborious T cell isolation and cloning. Alternatively, direct isolation and sequencing of MHC-associated peptides from cells has been limited by the overall low concentration of specific peptide bound to MHC molecules, and the highly polymorphic nature of the MHC molecules themselves. These challenges are being overcome by advances in high-throughput MHC-peptide binding studies, the bioinformatics of epitope prediction, and in particular, mass spectrometry.

With high-throughput peptide synthesis, direct MHC-peptide binding assays may now be performed on peptides that span

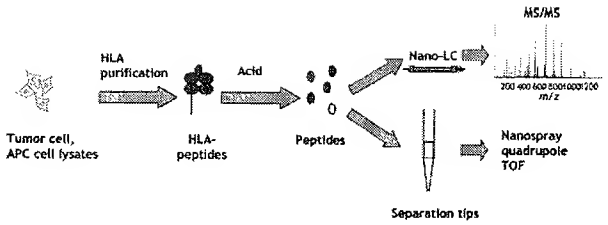

Figure 5. Identification of HLA-binding peptides by mass spectrometry. Peptide-MHC complexes are immunopurified from tumor cell (or antigen presenting cell, APC) lysates using antibodies directed against HLA molecules. Bound peptide mixtures are eluted in acid, and separated by nano-LC followed by MS/MS identification (top arrow). Alternatively, peptides may be directed applied to nanospray quadrupole TOF (bottom arrow), and predicted epitopes identified by fragmentation patterns.

target antigens, or using combinatorial peptide libraries.[107] Detailed analysis of peptide binding to common HLA alleles has resulted in the development of bioinformatics tools for epitope prediction.[108,109] Further limitations imposed by proteasomal cleavage patterns can enhance prediction of peptide epitopes. Multiple prediction models are available, primarily for MHC Class I-binding peptides, due to the more restrictive nature of the peptide binding groove. These systems include BIMAS,[110] SYFPEITHI,[111] MHCPEP,[112] and RANKPEP,[113] among others.

Advances in mass spectrometry have also greatly aided antigen identification. Original studies of direct peptide sequencing from purified MHC molecules from cell lines[114-116] demonstrated that the majority of peptides within the MHC grooves are derived from self-proteins, but required billions of cells for the source of antigen. More recent approaches to identify tumor-specific T cell epitopes have used capillary-scale chromatography and tandem mass spectrometry (Figure 5) (nanoLC–MS/MS).[117-120] To enhance sensitivity, nanospray quadrupole-TOF combined with Poisson algorithms[121] to specifically evaluate the likelihood of predicted peptides in unfractionated mixtures has markedly increased the sensitivity of the assay to the range of 1 copy number of peptide/cell in a murine influenza viral model, but is limited to predicted MHC-binding peptide epitopes.

Summary

The development of proteomic-based methods of cell lysate fractionation, phage display, protein microarrays, bioinformatics, and mass spectrometry is resulting in the rapid identification of both B and T cell tumor antigens. These have the potential for clinical diagnosis, identifying targets for immunotherapies, monitoring disease response, and to understand the breadth, scope, and impact of the immune response to cancer. As each new technology leads to the discovery of novel antigenic targets, systematic approaches of target validation and assessment of clinical applications, especially in the area of biomarkers and diagnostic testing, will need to be developed.

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Exhibit B
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Cheaper Chips Find a Good Fit with Hit Validation
Drug Discovery & Development - February 03, 2005
Protein microarrays promise to facilitate study of proteome interactions, and their use is growing despite proteins' inherent instability

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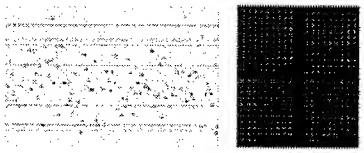
In November 2004, protein array technology hit a milestone: Invitrogen Corp., Carlsbad, Calif., released the world's first commercially available high-density human protein microarray. The ProtoArray, which contains more than 1,800 unique human proteins, represents a cross-section of gene families including pharmaceutically relevant protein classes such as kinases and membrane-associated, cell-signaling, and metabolic proteins.

The pharmaceutical industry's demand for protein arrays has been high, and for good reason, says Steven Bodovitz, PhD, principal and cofounder of BioPerspectives, San Francisco, and an expert on protein biochips, protein biomarkers, and proteomics. "Mass [spectrometric] studies are very good at discovering potential targets and biomarkers, but they're not so good at following up with validation," he says. "You need to be able to take your initial findings and then see the protein change, and follow up and test it over and over again in different tissues, at different time points, under different conditions. To achieve that, you need a lower-cost, higher-throughput screening technology, which is a fantastic fit for the protein biochip."

Indeed, some experts have long been saying that protein arrays could potentially surpass DNA microarrays in their scientific impact. But moving from expectation to reality with these arrays has proven to be a longer journey than first imagined when protein arrays came on the scene in the late 1990s. "It was first thought that protein biochips would just be an extension of DNA microarrays, and that hasn't exactly panned out," says Bodovitz.

That's because proteins have proven to be much trickier to work with in array format than their genomic counterparts. First of all, there are issues of stability. Membrane proteins, for example, make up the majority of potential drug targets, but they're particularly challenging to stabilize. Then there's the choice of immobilization technique, which determines how well the target protein presents itself to the capture agent, and the problem of nonspecific binding. And of course, proteins are inherently unstable outside their natural habitat of living cells, making them much more challenging than DNA to tag and manipulate.



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Comparative analysis of normal and cancer sera using the Schleicher & Schuell Serum Biomarker Chip. Scatter plots of protein abundance ratios comparing serum from breast cancer patients with serum from healthy, age- and gender-matched individuals. Serum proteins were labeled with Biotin-ULS and Fluorescein-ULS, pooled, and probed against the Serum Biomarker Chip. (Source: Schleicher & Schuell)

Despite these challenges, though, the protein array market continues to grow. What was a $122 million market in 2002 will jump to $545 million by 2008, predicts an August 2004 report, *Protein Biochips: Parallelized Screening for High-Output Biology*. The report was released jointly by BioPerspectives; Bachmann Consulting in Nesoddtangen, Norway; and the NMI Natural and Medical Sciences Institute at the University of Tübingen, Germany. "The industry has begun to make the transition from a few years ago, where there were a lot of grandiose expectations, to very specific, aggressive approaches to developing protein biochips," says Bodovitz.

Although Invitrogen has lately been methodically gobbling up competitors and was the first company to offer a human protein biochip, the market for protein arrays is unlikely to be nailed down by a few leading vendors in the way that Affymetrix virtually cornered the market on DNA microarrays. "The genome is basically a limited set of information. Once you have a DNA microarray that covers the whole human genome, there is not a lot of room for something else," says Bodovitz. "That won't be the case at all with protein biochips. You have the capture and the interaction sides, which are very different technologies, and no one's yet covering the whole proteome, so no one big company is dominating."

Bright Shiny Beads

With all of the challenges inherent in developing solid-surface arrays that can hold thousands of proteins, all with different properties, it's little wonder that another approach—protein-interaction assays in solution—is also drawing attention. A leader in this market is Luminex Corp., Austin, Texas, whose bead-based xMAP system can provide up to 100 assay results from a single drop of sample. Like the planar arrays,

click the image to enlarge

Luminex's bead-based protein arrays attach reagents to the surface of color-coded beads, which can be studied in suspension at a

A capture audience

What's the best approach to protein arrays? It depends on whom you ask, and what your goal is. Protein arrays can generally be broken down into two main categories: capture arrays and interaction arrays. Capture biochips use immobilized capture agents to capture target proteins, while interaction biochips have immobilized proteins or peptides which are used to identify functions or tease out interactions with other proteins or small molecules.

"For capture arrays, the big issue is content. In DNA microarrays, the oligo on the surface captures the target sequence and binds to it, but to capture a protein, you need a different, more complicated agent," says Bodovitz. "Historically, the problem has been that there aren't enough high-quality capture agents to populate a broad or high-density protein biochip platform. Antibodies don't work if they are denatured, for example."

Early solutions that appeared to have promise for rapidly generating research reagent antibodies don't appear to have panned out, so many companies are narrowing their sights to more focused content. "You organize a limited number of antibodies, say 30 or 40, maybe, sometimes more. The key is to give it a focus," says Bodovitz. "The most common focus is on cytokines. It's possible to cover a large number of the cytokine population on a biochip."

Luminex's protein arrays also attach capture reagents to the surface, in this case, a bead surface. Each bead is color-coded to distinguish what reaction is being demonstrated.

rate of 100 targets within a few seconds. (Source: Luminex)

"The majority of protein array tools out there use a flat-surface, two-dimensional array. We try to mimic as well as possible what's actually happening in the cell. Biology doesn't happen in a tube," says James Jacobson, PhD, Luminex's vice president of research and development. "The beads allow a lot of things to happen that are advantageous. Because they're small and in suspension, we can take advantage of very favorable reaction kinetics. We also have a very high sample throughput; 100 results in a few seconds is pretty fast."

Luminex has moved away from direct array sales and instead distributes them through a range of partnerships with companies like Bio-Rad Laboratories, Hercules, Calif., and Qiagen, Valencia, Calif., which provide add-on offerings like cytokine assays and assays for apoptotic markers and specific phospho-proteins, associated software, and reagents and kits that allow users to develop their own assays for the multiplexed environment.

That is an environment that interests many pharmaceutical scientists. "Multiplexing in protein arrays is promising, especially for cell signaling and signal transduction," says Eli Lilly's Myrtle Davis, DVM, PhD. "In those arenas, it's all about pathways, not just a single protein. We need to be able to identify a set of markers that indicate a particular modulation, not just one protein, and for that you need to see multiple protein interactions at one time."

"The best arrays now only have 100 antibodies or less," says protein array expert Michael Snyder, director of the Yale Center for Genomics and Proteomics, New Haven, Conn. "I think it will take some new technologies to measure thousands of things using antibody arrays or other sorts of capture reagents. I don't think we know yet what are the best capture reagents, [be they] aptamers, monoclonal antibodies, or single-chain antibodies." Snyder thinks there are ways of improving the specificity of those reagents, ways that have yet to be explored.

One of the leaders in the capture array market is Schleicher & Schuell (S&S), Keene, N.H., which in June 2004 released its S&S Serum Biomarker Chip, the first high-density antibody chip specific to serum biomarkers related to human cancers of every major organ. The chip uses a cisplatin labeling chemistry that tags small molecules to serum proteins to label each sample with two different tags. The samples are then pooled and probed against the antibody microarray in a competitive binding fashion.

"The translational cancer research community didn't really have an easy-to-use tool that did not require specialized training, so the serum biomarker chip was probably the first product that addressed that need. Researchers can now identify a pattern of the abundance of protein in a diseased individual versus a matched healthy person, and, for example, identify a molecular signature that acts as a surrogate end point rather than a clinical end point, 12 to 16 weeks before you would see tumor response to a therapy," says business development manager Robert Negm, PhD.

As little as 8 mL can be processed to discriminate the abundance of more than 120 cancer serum biomarker proteins between two individuals. S&S has taken great pains to eliminate nonspecific binding, says Negm, using its FAST Quant TH1/TH2 assay (an alternative to microplate ELISAs for the assaying of multiple cytokines) to demonstrate that the antibody is not binding nonspecifically to another protein in the serum sample.

Still, their antibody-based nature remains the biggest limitation for capture arrays, says Myrtle Davis, DVM, PhD, senior research scientist at Eli Lilly and Co., Indianapolis. "Antibody-protein interaction is a wonderful thing that we can exploit as a means to pull out proteins from complex mixtures, but we do know that it's extremely limited."

At this point, she says, the quality of the protein array depends on the quality of the antibody. "One of the questions about antibody arrays that I always ask every vendor is this: if they use a chemistry to bind antibody to a solid phase, for example, how can they be sure that the antibody is bound in a conformation that allows it to capture antigens?" she asks. "You want to have some quality control on well-to-well variation."

Like Snyder, she thinks improved capture reagents are an important goal. "We need some other capture chemistries to be defined, so that these arrays can start to be more useful to the protein community."

Also on Davis' wish list is a protein binding technology that is less vendor-specific. "Reagents are often very specific to the vendor, for example, and we've found that when you start to employ some of these technologies, you're tied to the vendor," she says. "If a company goes under, you've set up an entire assay system around a technology you can no longer use."

Interaction in action

In terms of interaction arrays, Invitrogen appears to be unchallenged. Within the last year, Invitrogen acquired Protometrix, Branford, Conn., the developer of the Yeast ProtoArray, precursor to the Human ProtoArray. It also licensed rights to specific fields of use for more than 30 patents in the area of protein microarray development from Zyomyx Inc.

Interaction arrays are "the wild card," says Bodovitz. "If you talk to most protein biochemists, their reaction is, 'This can't possibly work.' Protein biochemistry is notoriously finicky, and people usually highly optimize any one reaction they're studying. Now you're talking about doing thousands of biochemical reactions on a chip surface? First, you're only studying them under one condition, plus immobilization could also have an effect. This means your data could only be applicable to one set of condition, and it may not be representative of anything."

He put this question to Protometrix scientists before the Invitrogen acquisition. "They countered that when they compared the reactions on the chip versus taking proteins

Table of No Content

If you had a protein chip with no proteins on it, then you'd have the ProteinChip system from Ciphergen Biosystems Inc., Fremont, Calif. The ProteinChip array consists of a variety of preactivated, chemically treated surfaces, designed for expression profiling when you're not sure just what protein you're looking for. Its main application is biomarker discovery and assay.

The ProteinChip, says Kate Gilbert, Ciphergen's director of marketing, is ideally suited to de novo discovery. "A researcher may be looking at a disease biomarker or efficacy biomarker and trying to predict response, and in many cases, may not be really sure what protein is going to prove to be a good marker," Gilbert says. "If you have antibodies down there, you're only going to find the proteins you have antibodies for. This approach, on the other hand, allows you to discover any type of protein, providing that it will bind under the chromatographic conditions you've selected."

The ProteinChip uses broad conditions in order to capture as much of the proteome as possible. The process is simple, designed as a benchtop system usable by individual researchers. First, a biological sample is put on the chip, and subpopulations of proteins are captured, retained and purified directly on the chip by affinity capture. The ProteinChip Reader uses a laser to desorb the retained proteins into a time-of-flight mass spectrometer; and accompanying software records and presents the molecular weight of the proteins found.

individually and doing it in solution, they got the same kind of results," he says. "Immobilization, at least, has been controlled for and apparently has very little impact on results. If you can make an initial discovery through a fast, very broad screen, and it holds up in subsequent assays, that's a very powerful method."

"Our chips allow you to segregate proteins and give you the chance to see things you wouldn't see if you looked at one sample," says Gilbert.

Pharmaceutical scientists are also attracted to Invitrogen's interaction arrays for specificity profiling. "The techniques currently used to assess antibody specificity are relatively crude. Western blots and such will largely characterize an antibody as specific or nonspecific, but they'll fail to identify exactly what the cross-reactivity is," says Predki. "With microarray experiments, in about a half a day, you can not only assess specificity but can immediately determine cross-reactive proteins."